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MOLECULAR STRUCTURE OF THE HUMAN SKIN'S BARRIER: ELUCIDATION, FORMATION AND UTILIZATION

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Abstract

The main focus of this study is the permeation barrier of human skin. Original cryo-electron microscopic images from near native samples of human epidermis was used as the guiding reference to assemble *in silico* molecular models and also to assess the possibility of their presence in the barrier. Molecular dynamics and transmission electron microscopy simulations have been successively deployed to obtain models closer to a more naturally occurring state and attain simulated electron-microscopic images from them comparable to those from the real cryo-electron microscopy. The important questions that were investigated are: How is the barrier formation initiated? What are the transient structures involved and how do structural transformations advance during the formation process? What final structure does it form into?

The results show support for a hydrated and tightly folded cubic structure containing glycosylceramides as the starting structure, compatible with reference cryo-electron microscopy images from the topmost layer of the stratum granulosum. Furthermore it is shown how this structure has the ability to collapse into a lamellar structure after deglycosylation and dehydration. Subsequent stages of the development of the barrier are visible in the microscopic data from the lowermost layers of stratum corneum. The data show uniform and non-uniform fine striped patterns. We attempted to arrive at molecular structures that could explain the cryo-electron microscopy patterns. Multiple systems were tested and the ones with the best match were selected as the most plausible model. The same procedure was applied to determine the structure of the fully formed barrier, which is thought to be the major obstacle for permeation of different substances into the body. It is further demonstrated how an accurate molecular model of the skin's barrier structure can be utilized to predict some of its physical properties such as its permeability properties or thermotropic behaviour, revealing the capability of computer simulations in situations where the lab experiments are unwieldy or impossible.

List of publications

1. A Narangifard, L den Hollander, Christian L Wennberg, M Lundborg, Erik Lindahl, I Iwai, H Han, S Masich, B Daneholt, and L Norlén. Human skin barrier formation takes place via a cubic to lamellar lipid phase transition as analyzed by cryo-electron microscopy and em-simulation. *Experimental cell research*, 366(2):139–151, 2018, https://doi.org/10.1016/j.yexcr.2018.03.010.

2. Christian L Wennberg, Ali Narangifard, Magnus Lundborg, Lars Norlén, and Erik Lindahl. Structural transitions in ceramide cubic phases during formation of the human skin barrier. *Biophysical Journal*, 114(5):1116–1127, 2018, https://doi.org/10.1016/j.bpj.2017.12.039.

3. Ali Narangifard, Christian L. Wennberg, Lianne den Hollander, Ichiro Iwai, HongMei Han, Lundborg Magnus, Sergej Masich, Lindahl Erik, Daneholt Bertil, and Lars Norlén. Molecular reorganization during formation of the human skin barrier studied in situ. *Manuscript*

4. Magnus Lundborg, Christian L Wennberg, Ali Narangifard, Erik Lindahl, and Lars Norlén. Predicting drug permeability through skin using molecular dynamics simulation. *Journal of controlled release*, 283:269–279, 2018, http://dx.doi.org/10.1016/j.jsb.2018.04.005.

Additional publication

Magnus Lundborg, Christian L Wennberg, Ali Narangifard, Erik Lindahl, and Lars Norlén. Predicting drug permeability through skin using molecular dynamics simulation. *Journal of controlled release*, 283:269–279, 2018, https://doi.org/10.1016/j.jconrel.2018.05.026.

Contents

List of Abbreviations

CEMOVIS cryo-electron microscopy of vitreous sections.

cryo-EM cryogenic electron microscopy.

CTF contrast transfer function.

EM electron microscopy.

FFA free fatty acid.

MD molecular dynamics.

PBC periodic boundary conditions.

PSF point spread function.

SAXS small-angle X-ray scattering.

SC stratum corneum.

TEM transmission electron microscopy.

Chapter 1

Introduction

1.1 Skin

Skin is the largest organ of any terrestrial vertebrate. The skin, having immediate contact with the environment, has to cope with the severe ambience of dry land. Skin has reached a relative evolutionary perfection in isolating water inside and maintaining the intense hydration gradient between its two sides, even in the driest conditions without undergoing any irreversible change. It is also a protection against radiation, chemicals and harmful microorganisms.

It has been known that skin also works as an organ of sensation, regulation and even immunity by providing a dynamic substrate for commensal microorganisms [5]. It is evident how having more insight into the structure and formation of a healthy skin can benefit not only dermatology but also other areas of medicine and biology.

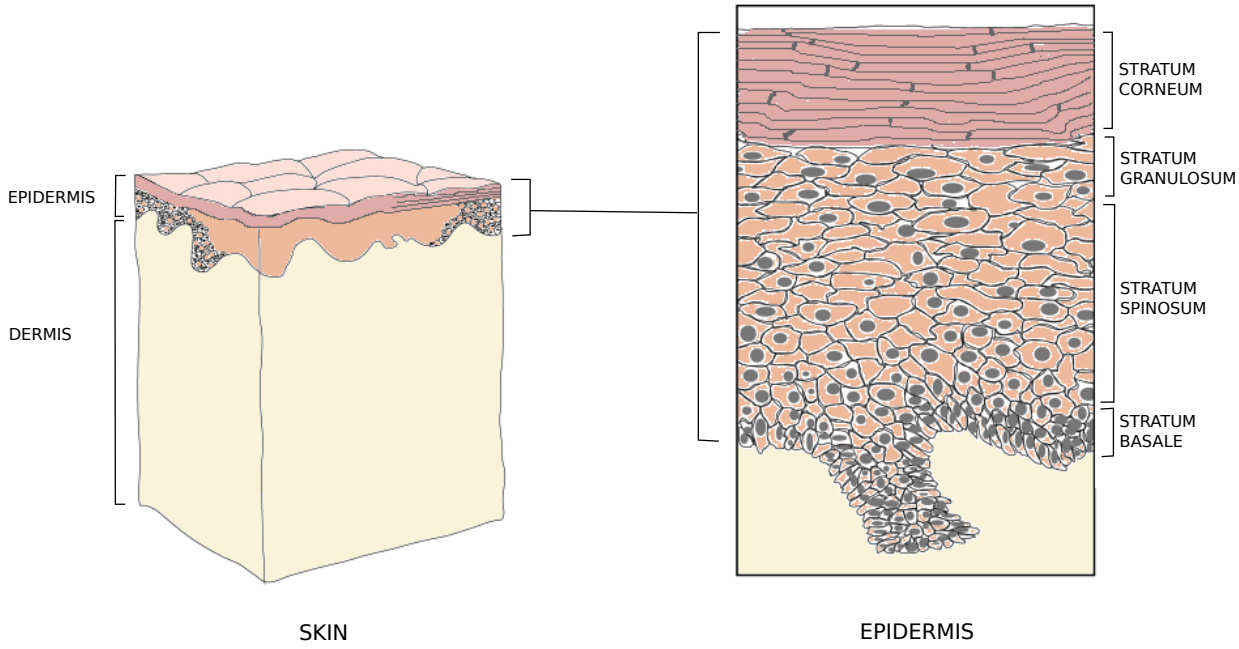

Figure 1.1: **Skin's structure** ˍ The stratum corneum, the topmost layer of the epidermis, is composed of flattened corneocytes.

1.2 Structure of the skin

There are three distinguishable layers in the human skin: *hypodermis* is the lowermost and consists of fat, nerves and hair roots - *dermis* which consists of dense connective tissue - and *epidermis* which is tightly connected to the dermis and is fabricated from densely packed keratinocytes. Keratinocytes are epithelial cells producing extensive amount of keratin proteins to form the unique physical properties and resilience of the skin. These cells lose their nucleus and organelles and go through a terminal differentiation. At this stage they are called corneocytes. As they get closer to the surface of the skin, the cells flatten and the lipids from their trans-Golgi network transform into a multilamellar structure and fill in the space between the corneocytes and together they make up the most impermeable part of the epidermis and skin, the *stratum corneum (SC)*. Corneocytes are eventually shed off the skin surface by desquamation as the new ones are being produced from beneath.

1.3 Significance of the lipid structure in the stratum corneum

1.3.1 Structure of the lipid matrix

As the first physical barrier to the world outside, SC is the shield protecting the body and by itself it bears some of the important features of the skin. It has been known from 1951 that it is the lipid content within the SC that gives its essential characteristic: the impermeability [6]. If we understand the structure of the lipid-packed areas (hereafter referred to as the *lipid matrix*), we may be able to explain the functionalities of the skin and have more intuition about the situations when they are not met. Furthermore, we will also be able to circumvent its characteristics to our advantage for example understanding how it can be used as a route of drug administration and, eventually, broadening the range of drugs that can be delivered locally or transdermally. These reasons have inspired scientists to determine the molecular structure of the lipid matrix for over three decades.

1.3.2 Formation of the lipid matrix

Knowing more about how the barrier is formed bears great significance for dermatology as many skin diseases, such as eczema, psoriasis and the ichthyosis, are characterized by barrier deficiency that may be caused by malformation of the lipid matrix. Furthermore, as the mature skin barrier lipid matrix cannot be guaranteed to represent an equilibrium structure, its molecular organization may depend on how it has been formed.

1.4 What is this thesis about?

This work focuses on three aspects of the skin barrier:

Elucidation

We have presented a new modified model for the molecular arrangement of a fully formed lipid matrix. The model not only retains its relative stability in the molecular dynamics (MD) simulations, its simulated electron microscopy images have a high resemblance to the real reference cryo electron microscopy data and it can replicate the trend in the permeability of different substances and solutions. The method developed for this purpose (cryo-electron microscopy combined with molecular dynamics simulation and electron microscopy simulation)

can potentially be used to solve any molecular structures from their cryo-EM representation in vitreous near-native tissue sections and knowledge about their composition [7].

Formation

We have tried to answer some critical questions about the formation process of the barrier's lipid multi-lamellar structures and how it could be set up from its primitive bilayer from based on cryo-EM observations from the deepest layers of the SC [2, 1]. A model is presented as the most probable for the transient structure before the lamellarization and it is demonstrated how such a structure can be transformed into lipid lamellae subsequent to deglycosylation and dehydration.

Utilization

We demonstrated how the model of the barrier can be utilized to predict some of its physical properties. Particularly we attempted to calculate the permeability of water through the lipid lamellae with promising results showing its potential as a predictive method for transdermal drug design [7]. In the next step (not included in this thesis), permeability of some reference compounds were calculated and compared to the experimental values and the effect of some penetration enhancers were explained [4].

Chapter 2

Background

2.1 The structure of the intercellular lipid matrix

It has been long known that the intercellular space of stratum corneum consists of abundant lipid molecules in the multi-layered structure. Breathnach et al. (1973) and Elias et al. (1975) performed the earliest studies suggesting a lamellar (laminated) structure for the lipid-rich intercellular areas in the SC using an electron microscope [8, 9]. Soon after it became evident that it is the lipid matrix that is responsible for the impermeability of skin, the debate about the molecular structure of the lipid lamellae began.

Striped patterns

A large number of different experimental methods were applied to resolve the structure of the intercellular lamellar lipid. Madison et al. (1987) used a secondary fixative during the sample preparation for electron microscopy to make a novel observation. For the first time they could reveal the structure of the intracellular pattern of the lipid corneocytes in the outer SC as well as more accurate pattern in the lamellar bodies [10]. As they demonstrated, in the lamellar bodies, there are alternating thick and thin dense bands existing between lucent bands. These bands are persisting in the intercellular lamellar layers of the deep levels of SC where the lamellar bodies are merging into the intercellular areas. The distance from a dense band to the next dense band is 9.7 nm. Distinctively in the upper level of the intercellular space, the dark and lucent bands are wider and measure 12.8 nm from a dense band to the next. All of these methods make use of sample staining and record the images in-focus and the contrast is created by the stain.

Early attempts using X-ray diffraction could not successfully replicate the patterns seen

in the electron microscopy probably due to extensive and adverse lipid extraction. White et al. (1988) speculated existence of an unknown protein inside the SC which has been cleared away in the preparation process which promotes the structures [11]. Later, Bouwstra et al. (1991) used small-angle X-ray scattering (SAXS) on the SC and concluded that lipid layers produce two distinctive periodicities: 6.4 nm and 13.4 nm [12]. Furthermore they showed that with increase in the water content, the unit cell with the repeat distance of 13.4 nm disappears; possibly related to the strong penetration-enhancing effect of water in high hydration levels, according to their speculation. Garson et al. (1991) used synchrotron radiation source to produce more accurate results. They only detected 4.5 and 6.5 nm thick bilayers but did not observe the 13.4 nm periodicity [13].

Molecular structure in the lipid matrix

Electron diffraction provided more information about the lipid organization in the intercellular space. Pilgram et al. (1999) could find evidence about the organization of the lipids in the barrier [14]. They recorded two hexagonally shaped and overlapping intensity maxima which they attributed to two orthorhombic crystalline packings of the ceramides existing in two consecutive layers. They addressed the lateral arrangement of the molecules rather than their longitudinal patterns. The interest to find the lateral arrangement of the molecules started almost thirteen years earlier by Landmann (1986) [15].

Different lateral arrangements of the lipids were proposed to match the observed data i.e. the periodicities observed persistently in the images and the molecule distances in the spectroscopies from as early as 1989 [16]. In 1994 Forslind introduced his *domain mosaic* model [17]. He proposed segregated lipids into bulks with a crystalline/gel phase separated by *"grain borders"* of lipids in fluid crystalline state. This model for the lateral lipid structure at each layer of the barrier attempted to explain the barrier function and the hydrophobic and hydrophilic pathways through it.

Bouwstra et al. (2001) used isolated SC lipids and X-ray diffraction to demonstrate the importance of free fatty acid in the matrix to form an orthorhombic rather than hexagonal configuration as it was reported before [18]. They proposed a molecular model for the matrix which can explain the pattern and periodicity observed by electron-microscopy of stained samples. FTIR and NMR spectroscopy were also used to gain information about the intermolecular distances between the lipid molecules inside the SC by Damien et al. (2010) [19] and Björklund et al.(2013) [20]. There are other models of the structure that have been proposed to fit skin's measurable nanoscale properties, i.e. the lamellar periodicity and the molecular content arrangement (Swartzendruber et al. (1989) [16], Hill and

Wertz (2003) [21], McIntosh (2003) [22], Schröter et al. (2009) [23] and Mojumdar et al. (2013) [24]).

In 2001 Norlén made some predictions about the structure of the layers in the barrier [25]: Any discontinuity in the phase of the structure will lead to compromised functionality of the barrier and the resilience against mechanical, chemical or thermal stress is achieved through a coherent gel phase, contradicting the domain mosaic model where there are two distinguishable coexisting phases of lipids in the layers. This model portrays the barrier as a macroscopically uniform entity in gel phase with no visible border between the phases; achieved by heterogeneity existing in the lipid composition, i.e. locally different distribution profile of the comprising lipids. Cholesterol molecules in a rather high concentration ($> 30\%$ molar) play key roles in the barrier capacity by (i) encouraging lamellar structures [26] and reducing the risk of development of possibly harmful "pores"; (ii) making the lamellar organization more stable to variations in the composition; (iii) enhancing the chain mobility of the lipids making them more "pliable" and mechanically resistant; (iv) extending the phase-transitional surfaces. Consequently the model presented consists of different possibilities that can coexist and transform to one another depending on the local conditions e.g. hydration level. Too much water promotes hairpin conformation in the ceramides and too little will make them splayed and create the long lamellar periodicity (in [12]) while interdigitation has not been excluded as a possibility for the short periodic patterns observed in the barrier. Norlén (2001) therefore renounces presence of a liquid crystalline structure in the barrier except before desquamation that is only limited to the upper layers.

Iwai et al. (2012) achieved a breakthrough in developing a method to resolve a more accurate model for the lipid matrix [27]. They used cryofixation and vitrification of the tissue without adding any artificial staining which leads to a better sample preservation. They then observed the sample with an electron microscope. Skin tissue is a transparent object for the electrons and the electrons are not attenuated (absorbed or scattered) enough to produce contrast. Therefore a meaningful image at the detector is unachievable by simple microscopy. It has been known that the image information is stored in the phase of the electrons rather than their amplitude; therefore detecting their energy would not help in forming an image. The similar problem in light microscopy has been solved by Frits Zernike in the early 1930s. His discovery states that simple manipulations of the wavefunction of the photons or electrons will lead to meaningful contrast in the magnitude of the waves and the information in the phase will reflect into the amplitude and will result contrast in the detector due to wave diffraction which is known as *phase contrast*. In electron microscopy phase contrast is created by imaging the sample a few micrometers out of focus. The drawback of imaging out of focus

is the artifact it introduces into the images making them difficult to be interpreted as if they are the result of a simple projection of the sample. Fortunately physics has explained and predicted this effect through a convolution with the *point spread function (PSF)* (or *optical transfer function (OTF)* in the Fourier space) which varies with the defocus value. The electron microscope adds other well-described artifacts which makes any interpretation of the image cumbersome. Iwai et al. (2012) used electron microscopy simulation in order to recreate the output image from any hypothetical atomistic model. They proposed a molecular organization that could replicate the images from a real microscopy through a computer simulation in three different defocuses. The computer simulator is called *TEM-simulator* [28] and developed to produce similar images of a transmission electron microscopy given all physical parameters and characteristics of the microscope during the microscopy session.

2.2 The formation

After the discovery of the main permeability barrier as the multi-lamellar lipid structures between corneocytes in the SC, the question that arises is how this structure is formed. There are several skin diseases associated with the malformation and therefore dysfunction of the barrier. Another importance of understanding the mechanism of the barrier's morphogenesis is how it can help gain more knowledge about the lipid matrix itself; as it is conjectured that lipid matrix is not an equilibrium structure and its molecular organization therefore dependent on how it has been formed [25]. A better understanding of the formation of the barrier will thus lead to a more insightful perception about its final structure. It is plausible that the excess lipid material is manufactured in the endoplasmic reticulum of the cells. The electron microscopy data provide evidence of a fine granular pattern of lipids next to the lamellar structure in the deep layers of the SC.

Landmann presented in 1986 [15] one of the earliest models about the barrier formation. The premise of this model was the creation of "lamellar bodies" from membrane fission or detachment from the trans-Golgi network of the keratinocytes. Lamellar bodies are thought to be unilamellar vesicles engulfing other flattened unilamellar vesicles recursively, called "lamellar body disks". They have been proposed to fuse with each other and with the plasma membrane at the stratum granulosum/stratum corneum interface and eventually form the continuous multilamellar membrane sheets observed in the intercellular space in the SC. There were some conflicts raised about the Landmann's model by Norlén in 2001 [29]: The formation starts with the continuous structures of trans-Golgi network and ends with another

continuous structure, the intercellular lipid matrix, via fission and the fusion of multiple discrete and disconnected vesicles and there are energy costs involved in these transformations. Furthermore, a discrete structure implies disrupted regularization and control of the formation process e.g. the hydration level during the formation. Norlén then suggests a new model without any topological disruption based on the concept of intersection-free membrane folding [30]. This model implies a continuous, coherent and dynamic medium that lipids undergo before they become part of the lamellar-structured barrier. Such structures have been observed or hypothesized existing in other biological systems such as parts of some intracellular membranes [31, 32, 33, 34]. Furthermore Norlén suggested that because of the minimized energy barrier for the transformation to a lamellar structure and the resemblance of its stained electron microscopic image to that of a known cubic membrane, this intermediate structure could be a folded membrane with cubic symmetry. Further investigations into lamellar bodies showed their interconnectivity in a tubuloreticular network supporting the proposition of topological continuity during the formation [35].

It has been shown that unlike stratum granulosum, where the polar glycosphingolipids and neutral lipids are almost equally abundant, the lipid matrix inside the stratum corneum contain no glycosphingolipids suggesting that they have been metabolized [36]. Subsequently it was suggested that this process (deglycosylation) is essential to the formation of the permeability barrier [37, 38].

2.3 Electron microscopy

Invented in 1933, electron microscopy surpassed conventional light microscopy in its resolution and enabled us to visualize how cell organelles function and see sub-cell structures and even some individual large proteins. It can achieve such a high resolution due to the quantum mechanical wavelength of a high-energy electron which can be up to 100,000 times higher than that of a visible light photon. Extreme specimen fixation is needed when they are being observed with such accuracy because small movements easily distort the resulting images. *Plastic embedding* [39] is the method widely used for specimen preparation due to its convenience, when a piece of tissue is the subject of examination. *cryo-electron microscopy of vitreous sections (CEMOVIS)* [40] is another preparation technique. It is less invasive and preserves the native environment in the specimen better although it is more laborious to perform.

2.4 Vitrification and CEMOVIS

Since the early 1980s, it has been known that despite thermodynamic predictions, water molecules in the specimen can be prevented to form ice crystals by rapidly submerging it into liquid nitrogen; known as vitrified water [41]. It has been an impactful discovery because forming ice in aqueous specimens prevented the scientists from observing them under the electron beam since the energy of the electrons is absorbed by the ice and they do not transmit to the other side of the specimen. This method has been used to make very thin films of vitrified aqueous solutions and enabled the influential and pervasive method of *cryo-electron microscopy for high-resolution structure determination of biomolecules in solution*, also known as single particle cryo-EM. In 2017 the Nobel prize in chemistry was awarded to the scientists involved in the discovery and invention of this method; Joachim Frank, Richard Henderson and Jacques Dubochet.

The problem with the thin film vitrification technique was that it could not be used to vitrify thick specimens from tissue, nor could the specimens be thinly sliced before freezing. Anything larger than 1 μm remained inaccessible until a new technique was introduced that allowed observing almost intact thinly sliced specimens from tissues. CEMOVIS [40] uses liquid nitrogen under high pressure (about 2000 bars) to vitrify a biopsy. Sections as thin as 30 nm are sliced off from the biopsy while kept at -140°C, then placed onto the holding grid and inserted into the electron microscope to be observed with a relatively low dose of exposure not to ruin the specimen.

Most of biological samples are transparent to photons and almost all are transparent to the electron beam. As a result of the remarkable work of Frits Zernik from the early 1930's [42] we are able to make contrast from a transparent object using rather simple interventions in the image formation process. In light microscopy a *phase plate* is inserted close to the focal plane before the image plane. Consequently, due to diffraction, the information in the phase of the wave eventuates in its amplitude and creates contrast in the resulting image. In the case of electron microscopy this effect is created by simply being out of focus during image previewing and data collection. Zernik won the Nobel Prize in physics in 1953 for his discovery.

2.5 Determining 3D structure

What is acquired by the electron microscope can often be approximated as a simple projection of the particle or structure under study especially if the specimen is very thin. If the image is not collected in-focus, an image deconvolution is needed to reverse the effect of

interference caused by being out of focus described by the *contrast transfer function (CTF)*. By gathering tomographic image series, 3D reconstruction can be performed to determine the structure. However, tomographic image series where we can determine the relative rotations between the projections is often hard to acquire especially when the aiming resolution is high and sometimes the structure under study does not produce a viable image that can be distinguished from the background noise. *Single particle electron microscopy* combined with image analysis provide tools to determine a particle's structure from thousands of projective images of the particle from random directions which exist in the sample solution. The images gathered by the CEMOVIS technique fall in a separate category though. CEMOVIS data cannot be studied from images from all of the observational angles since the specimen does not produce patterns distinguishable from noise at oblique angles and also because the sample is a tissue containing structures and solutions of various molecules and cannot be analysed with the single particle approach. A different method should be developed to analyze this type of images.

2.6 Electron microscopy simulation

Using high-energy electrons instead of photons introduces some differentiation between electron and light microscopy, especially with respect to how different electrons and photons interact with the molecules in the specimen and how the contrast is created. Quantum mechanics provides a highly accurate model of how the interaction occurs between the beam and the sample. Although the quantum mechanical equation is cumbersome to solve in a real case, there are methods to approximate the solution to the equation and predict how the beam is changed emitting out from the specimen. There also exists a model describing the interference occurring in the electrostatic and electromagnetic lenses, as well as models representing the collimator and detector to generate realistic looking images. These models, combined in a software package named *TEM-simulator* [28], compose a program simulating transmission electron microscopy, given the atomic structure of the specimen and the microscope's parameters, outputting predictions about the micrograph images. Important inputs are: dose, defocus, magnification, detector's pixel-size, etc. (figure 2.1).

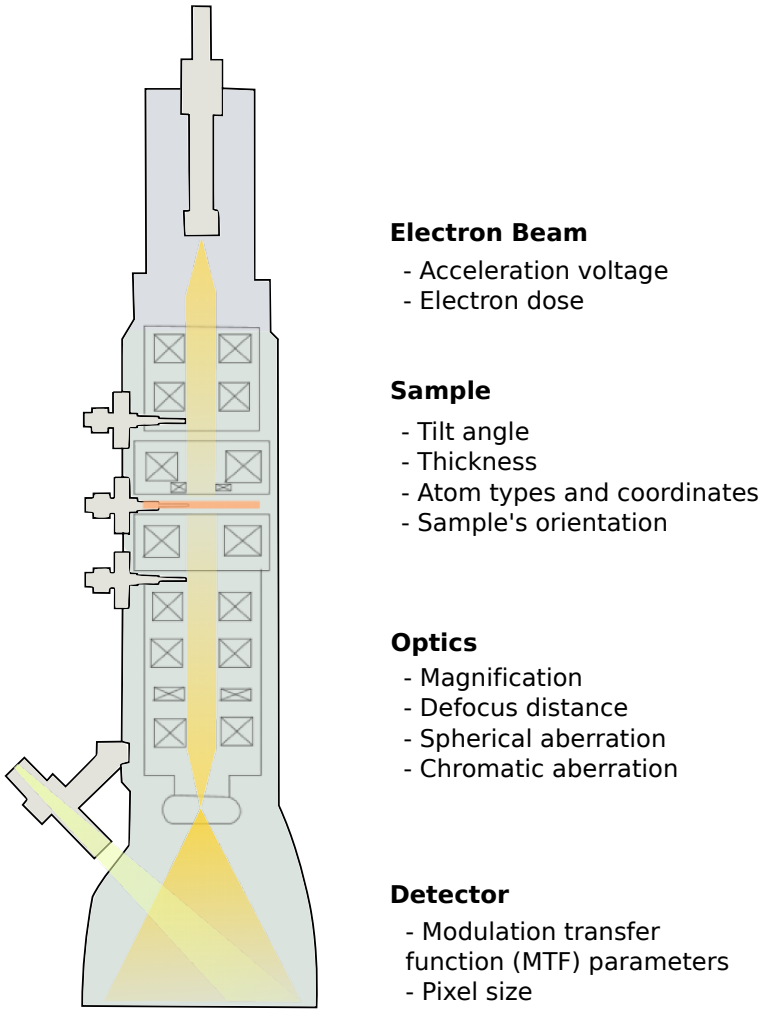

Figure 2.1: Schematic diagram of a transmission electron microscope and some important input parameters of TEM-simulator.

2.7 Molecular dynamics simulation

In classical mechanics, the movements of a system comprised of a set of rigid body particles can be predicted given the initial positions of the particles. Introducing global or mutual potentials, there will be conservative forces exerted on the particles. Let $V(x, y, z)$ be the summed potential energy for a particle as a function of its position, the conservative forces

add up to [1]:

$$\mathbf{F} = -\nabla V, \tag{2.1}$$

from which accelerations, velocities and positions can be calculated for small time steps. In 1738 Daniel Bernoulli tried to explain the behaviour of an ideal gas using classical mechanics on a set of particles. He postulated heat as the kinetic energy of the particles. Since then devotion of physicists such as Clausius, Maxwell, Boltzmann and Gibbs lead to formation of the *statistical mechanics*. They developed the formalism to explain macroscopic measurements from statistical microscopic characteristics of a system. Consequently it became evident that the Newtonian laws of motion; combined with electrostatic, bond and interatomic potentials; would be enough to explain macroscopic properties of a system. These potentials can be expressed by the following equation:

$$\begin{aligned} V = & \sum_{i<j} \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} && (i) \\ & + \sum_{i<j} \frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} && (ii) \\ & + \sum_{b \in bonds} \frac{1}{2} k_b (r_b - r_b^0)^2 && (iii) \\ & + \sum_{a \in angles} \frac{1}{2} k_a (\theta_a - \theta_a^0)^2 && (iv) \\ & + \sum_{d \in dihedrals} k_d (1 + \cos(n(\phi_d - \phi_d^0))) && (v) \end{aligned} \tag{2.2}$$

Each term represents a different type of potential:

(i) Electrostatic potential due to Coulomb forces between charged atoms

(ii) Lennard-Jones potential between atoms

(iii) Second-order approximation of the potential of covalent bonds between bonded atoms relative to their distance

(iv) Second-order approximation of the potential of bond angles for atoms with more than two bonds

(v) Sinusoidal approximation of the potential of dihedral angles

[1] In the atomic-level physics there is not any non-conservative force. Although energy can be dissipated to account for thermal dissipation to keep the temperature constant.

The idea of simulating the state of a molecular system was developed in the late 1950s [43]. With the advent and continuous improvement of the computing methods and hardware, it has since been optimized to deliver fast and accurate results. There are many constants in the equation 2.2 that must be determined for reciprocal inter/intra-molecular potentials for all of the atom pair types found in the system, before the equation can come into use. There are different approaches for this purpose to provide the best parameters that produce the most realistic simulation which are released as different *forcefields*. Forcefields are often optimized for particular sets of molecules and may therefore not produce accurate results for others and they might even have to be adjusted for a particular study.

Although the system under simulation should reflect a realistic and effective set of molecules, the size of the system is limited due to constraints such as the processing power. Moreover, the boundary condition of a simulation is largely influential on its result and should be carefully set. To solve these problems, molecular dynamics simulation is usually performed for a periodic system existing in a limited-sized periodic unit cell with periodic boundary conditions (PBC).

Chapter 3

Methods

In this chapter some technical details about each method mentioned in the previous chapter and how they were used in the different projects are discussed.

3.1 Electron microscopy data collection

The microscopic data used in all of the works had been collected prior to the analyses. Detailed information about sample preparation and data collection can be found in [44]. The steps for the preparation of the near-native samples for CEMOVIS can be summarized as: 1) take biopsy, 2) cut up into small pieces, 3) vitrify using high pressure freezer and store in liquid nitrogen, 4) trim with diamond trimming knife (Diatome) while kept at -150°C, section with the nominal thickness of 30 to 50 nm, 5) transfer to a pre-cooled mesh copper grid and press with stamping tool, 6) transfer the grid to a cryo-holder, place into the electron microscope and start the data collection.

EM data collection was performed with different magnifications resulting pixel sizes equal to 4.35 Å for some systems and 1.88 Å for the others. Nominal defocus values were ranging from -0.25 to -4 μm. Micrograph series were recorded with up to three different defocus values which enabled more detailed interpretations.

3.2 Molecular structure modeling

The first step for the analyses was to build the platform to make *in silico* models by arranging the atoms and molecules. For this purpose the framework *"Molar"* was created and kept

under development as the models needed new features [1].

Using Molar, geometric and stochastic parameterized models were defined. The models were built by placing different types of molecules at predetermined places sometimes with random dispositions and rotations. In case of the lamellar structures, these places were the points of stacked grids where the molecules were placed. For the cubic surfaces, first the triangulated sheets were created using spatial discretization followed by the *marching cubes* method [45]. Molecules were placed in the surface's triangles along their normal vectors. In order to have the correct molecule type distribution, before placing, a molecule is instantiated randomly with the probability according to the distribution aimed for each molecule type. There are several parameters controlling the models such as relative concentration of each molecule type, the number of water molecules, initial periodicity, etc.. More detailed description about how the systems were parameterized and made can be found in their corresponding articles and their supplementary material [2, 7, 1].

3.3 Molecular dynamics equilibration

Gromacs 5.0 [46, 47] was used as the MD simulation software and CHARMM36 [48, 49] was used as the forcefield in the all-atom simulations. After energy minimization, different stages of equilibration were run for the systems under different levels of restraint. As the last and longest stage, simulations without any restraints were run for at least 250 ns of simulation time, or until relative stability was achieved or until a selected set of properties of the system exceeded some thresholds. Particularly it was the *periodicity* of the system that was selected as an indicative parameter for both checking the stability of the system and distinguishing between different systems mainly because it has been a fairly reliable measurement that (i) in electron microscopy most likely remains constant subsequent to the artifacts added by the optics and detector of the microscope, (ii) is easily readable from the MD results, (iii) its stability is a sign of achieving, or approaching, a local equilibrium. Another purpose for MD simulation is to tune the distance between the atoms in the system based on what is recognized as physically natural. This is essential for the next stage, yielding realistic electron microscopy simulation from the systems.

[1] doi:10.5281/zenodo.1118401. Source code at: https://github.com/alinar/Molar

3.4 Electron microscopy simulation

In order for the systems to be comparable to electron microscopic data, they have to undergo electron microscopy simulation to generate images resembling the real data. First, the periodic box from MD is repeated in three dimensions to the size comparable to a real sample. TEM-simulator makes a potential map for the entire sample and virtually rotates the sample and takes a slice of its map for simulation. In the case of the lamellar structures, only one direction was studied; the direction at which the normal vector of the lamellar plates is perpendicular to the microscope's optical axis. Contrary to the lamellar structures, in the cubic systems there is not a particular orientation of the system that has priority over the other ones; therefore different three-dimensional orientations were chosen to cut the slices at from the virtual sample.

3.5 Data comparison

Having both the real and simulated electron microscopy (EM) images in hand we could discern similarities and conclude what system produces the closest EM simulation to the real ones. In the case of the lamellar systems, the indicative feature was the shape of the striped pattern they produce therefore one dimensional plots (intensity curves) were produced by integrating the pixel values along the lines of the striped image patterns, thereby aiding image discrimination. In the cubic systems, comparisons were done exclusively by eye.

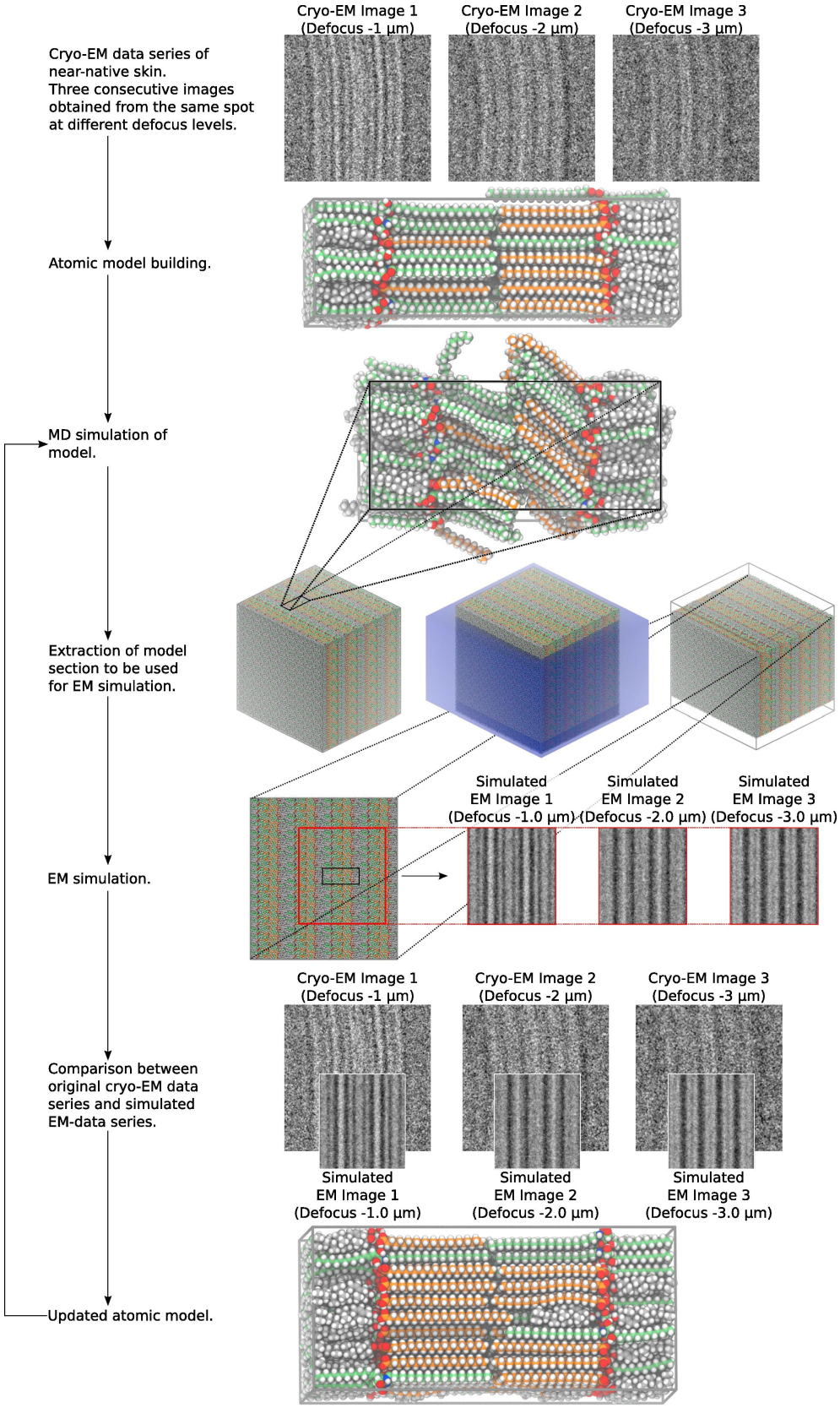

Figure 3.1: Illustration of the overall workflow used to elucidate the molecular structure of the different stages during the formation of the lipid barrier. (image from [7])

3.6 Refinement

To arrive at an optimized system which showed the best fit between the simulated and the real EM images, multiple systems were made by choosing variant combinations of values for the models' parameters. After MD and EM simulation the images were compared and estimations made about how the systems could be changed in order to achieve an optimized match. Thereafter, either more models with other parameters were built or more fundamental changes were applied to the models to generate more versatile systems to expand the searching range.

Chapter 4

Summary of Papers

4.1 Human skin barrier formation takes place via a cubic to lamellar lipid phase transition as analyzed by cryo-electron microscopy and EM-simulation

With the initiation of the formation of the lipid barrier in the topmost cell layers of the stratum granulosum, the lipids are synthesized in the endoplasmic reticulum and the Golgi apparatus of the cells. Excessive amounts of lipids with unknown level of hydration form a distinctive granular pattern in the electron microscopic images. Some of it form separate regions where the lipid molecules appear as uniform striped patterns with the periodicity of 5.0-5.5 nm. These granular and striped patterns abundantly appear alongside each other. This paper presents original cryo-EM data from in situ and near-native state samples and attempts to determine the system associated with the granular patterns as well as the system associated with the earliest lamellar structure in the process of the lipid barrier's formation.

The results showed compatibility of bicontinuous cubic structures with the observed EM patterns. Also some dehydrated lamellar phases agreed with the striped shape that appear next to the granular pattern.

4.2 Structural transitions in ceramide cubic phases during formation of the human skin barrier

In this paper we showed how a cubic structure containing skin glycosylceramides and water is stable in a coarse-grained MD simulation. The structure was built with the *gyroid* surface

shape and the lipid bilayer laid on the surface and the rest of the volume filled with water. After checking stability, deglycosylation and gradual dehydration was applied to the system. It has been suggested that deglycosylation of ceramides could facilitate releasing the lipid material out of the cells [50] and together with dehydration, lamellarization takes place. We could demonstrate how the system is destabilized and transforms into a lamellar structure only after deglycosylation and dehydration took place. The starting and ending structures were EM simulated and compared to real cryo-EM data from skin. We showed correlation between molecular lateral mobility in the surfaces and the curvature of the surface in the cubic structure. An interesting observation was clusters of splayed-chain ceramides as the system transforms and relaxes into a dehydrated stacked lamellar structure that might be showing the commencement of the transformation of the ceramides from hairpin into splayed conformation that is shown to exist in the fully formed barrier.

4.3 Molecular reorganization during formation of the human skin barrier studied *in situ*

Original cryo-EM images show two new striped patterns dominating the intercellular space of lowermost stratum corneum: (i) a symmetric and uniform striped pattern with periodicity of 2.0-2.5 nm, and (ii) an asymmetric two-band pattern with periodicity of 5.5-6.0 nm. Such small periodicities have not been recorded before and they are hypothesized to represent different end-stages of the formation of the barrier. Different possible structures were tested to explain these new patterns.

The results for the uniform striped pattern showed compatibility of a system with alternating molecule orientations at each layer, ceramides in splayed conformation and with interdigitated tail groups from the neighboring layers. Another system that showed compatibility was created only from monolayers of alternating-oriented cholesterols. For the asymmetric two-band patterns, a system composed of stacked asymmetric interdigitated bilayers with splayed ceramides and cholesterol mostly associated with the ceramides' sphingosine side had the best compatibility with the original cryo-EM data.

4.4 Human skin barrier structure and function analyzed by cryo-EM and molecular dynamics simulation

The structure of the fully formed permeation barrier was the focus of this paper. We used the high resolution cryo-EM data from Iwai et al. (2012) [27] and started with their proposed model: the lipid structure of stacked bilayers of ceramides in splayed conformation and cholesterol associated with sphingoid moiety of the ceramides and free fatty acid (FFA) associated with the ceramide fatty acid moiety. Primarily, all structures used for EM simulations were first subjected to a local equilibration using MD simulation. Further, we repeated the procedure with a more realistic composition of the compounds additionally including cholesterol sulfate, acyl ceramide, charged FFA, phytosphingosine based ceramides, etc. with their reported chain length distributions. The result model showed compatibility with the EM patterns and close to the correct periodicity. Moreover, to make the simulated images even more similar to the cryo-EM data, some refinement performed on the model; most importantly, water molecules were introduced to the head group regions and different cholesterol distributions between the two sublayers was tested. The refined model has overall relative density of 1:1:1 between different types of ceramides, cholesterols and fatty acids; 75% of the cholesterol is associated with the sphingoid moiety of the ceramides and the rest of cholesterol with the fatty acid moiety; 5% of the lipids is acyl ceramide (CER EOS); and water content is 1/3 water molecule per lipid and located in the headgroup region.

Using further simulations together with calculations, permeability of water was estimated for different studied models for which the proposed model showed one of the lowest values. The result was then compared to the real permeability measurements. Additionally, thermotropic behaviour of the structure was MD simulated and compared with the reported phase changes in the lipid bilayer and showed some structural changes at the temperatures where thermotropic transitions take place. These steps demonstrate how simulation using an accurate molecular model can be used to predict physical properties of a system.

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